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(54) Title: METHODS FOR INHIBITING CELL PROLIFERATION BY INHIBITING THE MITOGENIC ACTIVITY OF MACROPHAGE MIGRATION INHIBITORY FACTOR

(57) Abstract

This invention is directed to methods of inhibiting cell proliferation by inhibiting the mitogenic activity of macrophage migration inhibitory factor ("MIF"). Inhibitory nucleic acids directed against MIF inhibit cell growth in both cells induced by growth factors and transformed (malignant) cells that express oncogenes.

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5 ## METHODS FOR INHIBITING CELL PROLIFERATION BY INHIBITING THE MITOGENIC ACTIVITY OF MACROPHAGE MIGRATION INHIBITORY FACTOR

This application is a continuation-in-part of U.S.
10 Serial No. 08/340,826, filed November 16, 1995, incorporated herein by reference.

BACKGROUND OF THE INVENTION

This invention relates in general to the fields of
15 cellular and molecular biology and, in particular, to methods of inhibiting the growth of cells by inhibiting the mitogenic activity of macrophage migration inhibitory factor.

The control of cell growth is of interest to scientists in the understanding of normal physiological
20 activity, such as erythropoiesis and wound healing, and of pathological conditions, such as cancer. In this regard, the relationship between peptide growth factors and oncogenes, genes whose expression causes cells to become cancerous, has caught the attention of scientists. (See, e.g., M. Sporn et
25 al., Principals of Cancer Biology, Chapter 3 in Cancer -- Principals & Practice of Oncology, 2nd Ed., V.T. DeVita, S. Hellman and S.A. Rosenberg, Eds., J.B. Lippencott Company, Philadelphia (1985).) Certain mechanisms of cell proliferation in cancer appear to mimic the growth factor-
30 induced mitogenic pathway. Researchers have found, for example, that certain oncogenes, such as src, encode a protein kinase activity that resembles the protein kinase activity induced by certain growth factors. Studies have also shown that some peptide growth factors, such as PDGF, induce the
35 expression of oncogenes; and certain malignant cells require less stimulation by growth factors than normal cells to proliferate.

Peptide growth factors act by binding to receptors on the cell surface. From there, the induction of mitogenesis

involves a complex and only partially understood sequence of
intra-cellular signals and events. Growth factors induce the
expression of many genes, some of which are activated within
minutes, called primary response genes, and others which are
5   activated after a few hours, called delayed early response
genes. (See, e.g., Lanahan et al., (1992) *Molec. and Cell
Biol.*, 12:3919-3929.) The function of many of the proteins
encoded by these genes is unknown.

Among the many genes induced by these growth factors
10 is one encoding macrophage migration inhibitory factor
("MIF"). MIF was originally identified as a lymphokine
secreted by T cells at a site of infection. Upon contact with
monocytes, MIF causes them to stick to the blood vessel
endothelium where the cells force themselves through the
15 vessel lining into the surrounding tissue. There, monocytes
may differentiate into macrophages. Since the discovery that
T cells produce MIF, it was discovered that the protein is
synthesized by non-immune cells, for example, in the
developing eye lens, where its function is unknown. (G.
20 Wistow et al., (1993) *Proc. Natl. Acad. Sci., USA*, 90:1272-
1275.

There exists a need in the art for methods of
intervening in the mitogenic pathway induced by growth factors
and already active in cancer cells.

25

SUMMARY OF THE INVENTION
This invention satisfies this need and provides
related advantages as well by demonstrating that one of the
genes induced by growth factors, macrophage migration
30 inhibitory factor, or "MIF," is involved in cell proliferation
and that inhibition of MIF expression inhibits both peptide
growth factor-induced and transformed cell proliferation.

This invention provides methods useful for
inhibiting the growth of a cell involving the step of
35 inhibiting the mitogenic activity of MIF in the cell. The
methods can be used both *in vitro* to control growth of
transformed cells or cells stimulated growth factors, or the
method can be used *in vivo* in therapeutic methods.

According to one embodiment of the invention, the step of inhibiting the mitogenic activity of MIF involves providing the cell with an inhibitory nucleic acid that inhibits expression of MIF. The inhibitory nucleic acid can be a sense or an antisense nucleic acid that inhibits transcription of the MIF gene or translation of MIF mRNA. Antisense nucleic acids can be directed, for example, against a target sequence in the sense strand of the MIF gene, a target sequence spanning the boundary between an intron and an exon of MIF pre-mRNA, or, in a preferred embodiment, a target sequence in MIF mRNA. The antisense nucleic acid also can be a ribozyme that cleaves MIF mRNA. This invention also provides isolated forms of these inhibitory nucleic acids.

This invention contemplates delivering inhibitory nucleic acids to the cell by introducing an expression vector having an expression control sequence operatively linked to a nucleic acid sequence encoding the inhibitory nucleic acid. It also contemplates contacting the cell with the inhibitory nucleic acid, for example, by administration to the external milieu of the cell.

According to another embodiment of the invention, the step of inhibiting MIF activity involves inhibiting the binding of MIF to retinoblastoma ("Rb") protein.

This invention is also directed to therapeutic methods useful for inhibiting the growth of a malignant cell in an individual involving administering to the individual a therapeutically effective amount of an agent that inhibits the mitogenic activity of MIF in the malignant cell. The agent can be an inhibitory nucleic acid or a compound that interferes with MIF binding to Rb protein. In a preferred embodiment, the agent is injected into a tumor mass. This invention also provides pharmaceutical compositions having an agent that inhibits the mitogenic activity of MIF and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 presents results of PAGE on cell extracts from NIH 3T3 cells grown in the presence and absence of PDGF

4

and TGFβ1. Treatment of serum-free NIH 3T3 cells with either
PDGF or TGFβ1 results in stimulation of MIF expression.

Fig. 2 shows stimulation of the human MIF gene
promoter by PDGF and TGFβ1. NIH 3T3 cells were stably
5   transfected with plasmid containing the human MIF gene
promoter (V. Paralkar and G. Wistow, (1994) Genomics 19:48-51)
cloned upstream of the bacterial reporter gene chloramphenicol
acetyltransferase (CAT). Treatment of these cells resulted in
four-fold induction of CAT activity over serum-free control in
10   the presence of serum and 2-3 fold in the presence of TGFβ1
and PDGF, respectively. Similar results were seen in
transient transfection experiments. CAT activity relative to
serum-free control is shown as mean values + standard
deviation for three individual measurements.

15   Figs. 3A-3B show the effect of treatment with
antisense MIF oligonucleotide in abolishing cell proliferation
and synthesis of MIF protein.

Fig. 3A shows ^{3}H-thymidine incorporation in NIH 3T3
cells treated with growth factors and antisense or control
20   oligonucleotides. Cells were grown in the presence of 0.2%
serum and then exposed to (a) no additional treatment,
(b) PDGF (5 ng/ml), (c) PDGF + aMIF1 (10 μM), (d) PDGF + sMIF1
(10 μM), (e) PDGF + aMIF2 (10 μM), (f) PDGF + 1FIMa, (g) TGFβ1
(10 ng/ml) and (h) TGFβ1 + aMIF1. Antisense treated cells
25   recovered, i.e., they regained the ability to grow after
washing with fresh medium: (i) control cells were grown for
48 hr in 10% serum, (j) cells were first treated with
PDGF + aMIF1 for 24 hours as in part (c) then medium was
replaced with 10% serum for a further 24 hours. ^{3}H-thymidine
30   incorporation is shown relative to value for lane (a). Each
point represents mean ± standard deviation for three
measurements. The assay was repeated with multiple
preparations of oligos synthesized on two different Applied
Biosystems synthesizers giving similar results.

35   Fig. 3B shows a western blot of MIF protein in
NIH 3T3 cells grown in the presence of PDGF and TGFβ1 and
presence or absence of aMIF1 using antisera to human MIF.
Serum-free NIH 3T3 cells were cultured in the presence of PDGF

5

(10 ng/ml) or TGFβ1 (15 ng/ml) and presence or absence of
aMIF1 (10 μM) overnight. Cells were lysed at 4°C in a buffer
containing 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.6, 2 mM EDTA,
1 mM PMSF, 20 μg/ml aproteinin, 20 μg/ml leupeptin. Cell

5   lysates were clarified by centrifugation and their protein
concentration determined. Protein extracts (10 μg/treatment)
were subjected to SDS PAGE (Novex, San Diego, CA), transferred
to nitrocellulose filters and analyzed by western blotting
with chicken anti-human MIF antiserum. This antiserum was

10   raised as an IgY fraction in chickens immunized with a fusion
protein of human MIF and maltose binding protein.
Visualization was by enhanced chemiluminescence (Amersham,
Arlington Heights, IL), using goat anti-chicken immunoglobulin
secondary antibody (Vector labs).

15   Figs. 4A-4B demonstrate that MIF acts near the G1/S
boundary and interacts with Rb protein.

             Fig. 4A shows a western blot of cyclins in NIH 3T3
cells grown in the presence of PDGF and presence or absence of
aMIF1 using anti-cyclin antibodies. Non-specific bands are Ig

20   bands from immunoprecipitation.

             Fig. 4B shows a flow cytometry analysis of aMIF1
treated cells: a) control NIH 3T3 cells in the absence of
serum; b) serum-free cells grown in the presence of 5 ng/ml
PDGF; c) PDGF-treated cells in the presence of aMIF1.

25   Percentages of cells in G1, S and G2/M phases of the cell
cycle are indicated. Cells were grown as described herein.
Flow cytometry was performed as described in V.V. Ogryzko et
al. (1994) *J. Virol.* 68:3724, using a FACscan flow cytometer
equipped with a doublet discrimination (Becton-Dickinson).

30   Fig. 5 demonstrates that constitutive expression of
mouse MIF shortens G1 and allows cell proliferation
independent of serum. It shows results of: a) flow cytometry
of NIH 3T3 cells stably transformed with recombinant mouse MIF
cDNA grown in 10% serum; (b) control cells in serum; c) MIF-

35   constitutive cells in serum-free medium; (d) control cells in
serum-free medium.

             Fig. 6A shows a western blot of MIF in
immunoprecipitates of NIH 3T3 cell lysates using antisera to

cell cycle components which control G1/S transition. Rabbit
immunoglobulin-derived bands from the immunoprecipitation were
detected in all lanes. Lane C shows results for western blot
of anti-Rb antibody in the absence of cell lysate as control.
5   Lane 1 shows western blot of proteins immunoprecipitated with
agarose conjugated antiserum to Rb, retinoblastoma protein;
Lane 2 shows western blot of protein immunoprecipitated with
anti-cyclin E in the presence of agarose conjugated protein A;
Lane 3 shows western blot of proteins immunoprecipitated with
10 agarose conjugated anti-CDK2. All lanes were subjected to
western analysis with chicken anti-human MIF.

   Fig. 6B shows a western blot of MIF in proteins
immunoprecipitated by agarose conjugated anti-Rb under non-
reducing conditions. As in part (a), lane C is control
15 without cell lysate while lane 1 contains anti-Rb
immunoprecipitate. The immunoglobulin-derived bands now
migrate at larger size appropriate for intact antibodies. MIF
reactive protein in the anti-Rb immunoprecipitate migrates at
a size appropriate for a homodimer under non-reducing
20 conditions.

            DESCRIPTION OF THE SPECIFIC EMBODIMENTS
   This invention is directed to new methods of
inhibiting mitogenesis in a cell. The invention results from
25 the discovery that MIF has hitherto unrecognized mitogenic
functions and that the inhibition of MIF activity in a cell
results in the inhibition of cell proliferation. In
particular, mitogenesis normally induced by stimulation with
growth factors, such as Platelet-Derived Growth Factors (PDGF)
30 and Transforming Growth Factors-ß (TGF-ß), and mitogenesis in
cells transformed with the oncogenes *ras*, *raf* and *sis*, are
inhibited when MIF activity is inhibited with antisense
polynucleotides.

   PDGF and TGF-ß1 act through different signaling
35 pathways: tyrosine kinase receptors and serine/threonine
kinase receptors, respectively. Oncogenes *ras*, *raf* and *sis*
act as components early in the signalling pathways that lead
to cell division. The fact that inhibition of MIF halts cell

division in all these pathways indicates that MIF acts late in
the cell cycle after these pathways converge.

Results described herein show that MIF binds to the
retinoblastoma ("Rb") protein in the cell and is required for
entry of the cell into the S phase of the cell cycle. (See,
e.g., Wiman, (1993) *FASEB J.*, 10:841-845 and Cobrinik et al.,
(1992) *Trends Biochem. Res.*, 17:312-315.) Inhibiting MIF
activity arrests cells in G1 phase, and they cease
proliferation.

Accordingly, this invention provides methods that
result in the inhibition of cell growth involving inhibiting
the mitogenic activity of MIF in a cell, both *in vitro* and *in
vivo*. The inhibition of MIF activity of cells *in vitro* is
useful as a research tool to investigate the complex sequence
of events that regulate the cell cycle. The inhibition of MIF
activity in cells *in vivo* is useful in therapeutic methods for
inhibiting the growth of malignant cells.

In vitro, the methods of this invention are
applicable to any culture cells whose growth has been
stimulated by growth factors, such as, for example, PDGF,
TGF-β, Fibroblast Growth Factor ("FGF") or the Insulin-like
Growth Factors ("IGF"). It is also applicable to cells from
transformed cell lines, including cells transformed by the
introduction of oncogenes.

In vivo, the methods of this invention are
applicable, for example, to malignant cells. In particular,
this invention contemplates inhibiting the growth of cells
whose malignancy involves uncontrolled progression through S
phase, including those cells expressing oncogenes such as *c-
ras*, *c-raf* and *c-sis*. The methods of this invention are also
applicable to inhibiting the growth of cells *in vivo* whose
growth is induced by growth factors.

As used herein, "MIF" or "macrophage migration
inhibitory factor" means the human protein having lymphokine
activity and having the DNA sequence [SEQ ID NO:1] and deduced
amino acid sequence [SEQ ID NO:2] presented in Table I, its
alleles and any mammalian cognates of it. The sequence in
Table I is from V. Paralkar and G. Wistow, (1994) *Genomics*,

19:48-51 (GenBank Accession No. L19696). "#" indicates the
transcription start site. "*" indicates the beginning and end
of introns. "@" indicates the polyadenylation site (AATAAA).
The sequence of other MIF cDNAs are also known. (See, e.g.,
the following MIF sequences in GenBank: HSMMIHFA; HUMLMIF;
HUMMIFA; MMMMIHFA; MUS10KMIF; CHKLMIF; HUMGIA; and MUSGIA.
The last two are identified as glycosylation-inhibiting
factor.)

<div align="center">TABLE I</div>

```
  1 CTGCAGGAAC CAATACCCAT AGGCTATTTG TATAAATGGG CCATGGGGCC TCCCAGCTGG
 61 AGGCTGGCTG GTGCCACGAG GGTCCCACAG GCATGGGTGT CCTTCCTATA TCACATGGCC
121 TTCACTGAGA CTGGTATATG GATTGCACCT ATCAGAGACC AAGGACAGGA CCTCCCTGGA
181 AATCTCTGAG GACCTGGCCT GTGATCCAGT TGCTGCCTTG TCCTCTTCCT GCTATGTCAT
241 GGCTTATCTT CTTTCACCCA TTCATTCATT CATTCATTCA TTCAGCAGTA TTAGTCAATG
301 TCTCTTGATA TGCCTGGCAC CTGCTAGATG GTCCCCGAGT TTACCATTAG TGGAAAAGAC
361 ATTTAAGAAA TTCACCAAGG GCTCTATGAG AGGCCATACA CGGTGGACCT GACTAGGGTG
421 TGGCTTCCCT GAGGAGCTGA AGTTGCCCAG AGGCCCAGAG AAGGGGAGCT GAGCCACGTTT
481 GAACCACTGA ACCTGCTCTG GACCTCGCCT CCTTCCTTCG GTGCCTCCCA GCATCCTATC
541 CTCTTTAAAG AGCAGGGGTT CAGGGAAGTT CCCTGGATGG TGATTCGCAG GGGCAGCTCC
601 CCTCTCACCT GCCGCATGAC TACCCCGCCC CATCTCAAAC ACACAAGCTC ACGCATGCGG
661 GACTGGAGCC CTTGAGGACA TGTGGCCCAA AGACAGGAGG TACAGGGGCT CAGTGCGTGC
721 AGTGGAATGA ACTGGGCTTC ATCTCTGGAA GGGTAAGGGG CCATCTTCCG GGTTCACCGC
781 CGCATCCCCA CCCCCGGCAC AGCGCCTCCT GGCGACTAAC ATCGGTGACT TAGTGAAAGG
841 ACTAAGAAAG ACCCGAGGCG AGGCCGGAAC AGGCCGATTT CTAGCCGCCA AGTGGAGAAC
901 AGGTTGGAGC GGTGCGCCGG GCTTAGCGGC GGTTGCTGGA GGAACGGGCG GAGTCGCCCA
961 GGGTCCTGCC CTGCGGGGGT CGAGCCGAGG CAGGCGGTGA CTTCCCCACT CGGGGCGGAG
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<div align="right">*f*</div>

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1021 CCGCAGCCTC GCGGGGGCGG GGCCTGGCGC CGGCGGTGGC GTCACAAAAG GCGGGACCAC
1081 AGTGGTGTCC GAGAAGTCAG GCACGTAGCT CAGCGGCGGC CGCGGCGCGT GCGTCTGTGC
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                                       M  P  M  F  I  V  N  T  N
1141 CTCTGCGCGG GTCTCCTGGT CCTTCTGCCA TCATGCCGAT GTTCATCGTA AACACCAACG
```

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       V  P  R  A  S  V  P  D  G  F  L  S  E  L  T  Q  Q  L  A  Q
1201 TGCCCCGCGC CTCCGTGCCG GACGGGTTCC TCTCCGAGCT CACCCAGCAG CTGGCGCAGG
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       A  T  G  K  P  P  Q  *
1261 CCACCGGCAA GCCCCCCCAG GTTTGCCGGG AGGGGACAGG AAGAGGGGGG TGCCCACCCG
```

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1321 ACGAGGCGTT CCGCGCTGGG AGCTGGGGAG GCGACTCCTG AACGGAGCTG GGGGGCGGGG
```

```
1381 CGGGGGGAGG ACGGTGGCTC GGGCCCGAAG TGGACGTTCG GGGCCCGACG AGGTCGCTGG
```

```
                          *  Y  I  A  V  H  V  V  P  D  Q
1441 GGCGGGCTGA CCGCGCCCTT TCCTCGCAGT ACATCGCGGT GCACGTGGTC CCGGACCAGC
```

```
       L  M  A  F  G  G  S  S  E  P  C  A  L  C  S  L  H  S  I  C
1501 TCATGGCCTT CGGCGGCTCC AGCGAGCCGT GCGCGCTCTG CAGCCTGCAC AGCATCGGCA
```

```
       K  I  G  G  A  Q  N  R  S  Y  S  K  L  L  C  G  L  L  A  E
```

9

1561 AGATCGGCGG CGCGCAGAAC CGCTCCTACA GCAAGCTGCT GTGCGGCCTG CTGGCCGAGC

          R   L   R   I   S   P   D   R*
1621 GCCTGCGCAT CAGCCCGGAC AGGTACGCGG AGTCGCGGAG GGGCGGGGGA GGGGCGGCGG

                                                                      * V
1681 CGCGCGGCCA GGCCCGGGAC TGAGCCACCC GCTGAGTCCG GCCTCCTCCC CCCGCAGGGT

          Y   I   N   Y   Y   D   M   N   A   A   N   V   G   W   N   N   S   T   F   A
1741 CTACATCAAC TATTACGACA TGAACGGGGC CAATGTGGGC TGGAACAACT CCACCTTCGC

1801 CTAAGAGCCG CAGGGACCCA CGCTGTCTGC GCTGGCTCCA CCCGGGAACC CGCCGCACGC

                                                    €
1861 TGTGTTCTAG GCCCGCCCAC CCCAACCTTC TGGTGGGGAG AAATAAACGG TTTAGAGACT

1921 AGGAGTGCCT CGGGGTTCCT TGGCTTGCGG GAGGAATTGG TGCAGAGCCG GGACATTGGG

1981 GAGCGAGGTC GGGAAACGGT GTTGGGGGCG GGGGTCAGGG CCGGGTTGCT CTCCTCGAAC

2041 CTGCTGTTCG GGAGCCCTTT TGTCCAGCCT GTCCCTCCTA CGCTCCTAAC AGAGGAGCCC

2101 CAGTGTCTTT CCATTCTATG GCGTACGAAG GGATGAGGAG AAGTTGGCAC TCTGCCCTGG

2161 GCTGCAG

This invention contemplates inhibiting MIF activity in a cell by, for example, inhibiting MIF expression with inhibitory nucleic acids or by inhibiting the binding of MIF to Rb protein.

According to certain embodiments of this invention methods for inhibiting the mitogenic activity of MIF in a cell involve providing the cell with an inhibitory nucleic acid that inhibits expression of MIF. Inhibitory nucleic acids are single-stranded nucleic acids which can specifically hybridize to a target nucleic acid sequence. Typically, hybridization will occur when there is at least about 55% complementarity over a stretch of at least 14-25 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See M. Kanehisa, (1984) Nucleic Acids Res. 12:203.

By binding to the appropriate target sequence, an RNA-RNA, DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are usually complementary to the sense or coding strand of the gene. Recently, approaches for use of "sense" inhibitory nucleic acids have also been developed. The term "inhibitory nucleic acids" as used herein, refers to both "sense" and "antisense" nucleic acids.

By binding to the target nucleic acid, the
inhibitory nucleic acid can inhibit the function of the target
nucleic acid. This could, for example, be a result of
blocking DNA replication or transcription; interfering with

5   processing of, poly(A) addition to, or translation of mRNA; or
promoting inhibitory mechanisms of the cells, such as
promoting RNA degradation. Inhibitory nucleic acid methods
therefore encompass a number of different approaches to
altering expression of specific genes that operate by

10   different mechanisms. These different types of inhibitory
nucleic acid technology are described in C. Helene and J.
Toulme, (1990) *Biochim. Biophys. Acta.*, 1049:99-125, which is
referred to hereinafter as "Helene and Toulme".

Inhibitory nucleic acid approaches can be classified

15   into those that target DNA sequences, those that target RNA
sequences (including pre-mRNA and mRNA), those that target
proteins (sense strand approaches), and those that cause
cleavage or chemical modification of the target nucleic acids.

Approaches targeting DNA fall into several

20   categories. Nucleic acids can be designed to bind to the
major groove of the duplex DNA to form a triple helical or
"triplex" structure. Alternatively, inhibitory nucleic acids
are designed to bind to regions of single stranded DNA
resulting from the opening of the duplex DNA during

25   replication or transcription. *See* Helene and Toulme.

Accordingly, this invention is directed to methods
in which the inhibitory nucleic acid is directed against a
target DNA sequence in the sense strand of the MIF gene. In
particular, the target DNA sequence can span the site of

30   initiation of transcription, nucleotide number 1077 of SEQ ID
NO:1.

More commonly, inhibitory nucleic acids are designed
to bind to mRNA or mRNA precursors. Inhibitory nucleic acids
are used to prevent maturation of pre-mRNA. Inhibitory

35   nucleic acids can be designed to interfere with RNA
processing, splicing or translation.

One approach is to target the inhibitory nucleic
acids to U snRNP to thereby prevent the formation of

functional spliceosomes. Alternatively, inhibitory nucleic
acids complementary to sequences at the boundary between
introns and exons of the gene can be used to interfere with
cleavage of pre-mRNA and ligation of exons.

5   Accordingly, this invention is directed to methods
in which the inhibitory nucleic acid is directed against a
sequence spanning the boundary between an intron and an exon
of MIF pre-mRNA. Table I indicates the location of introns
and exons in the MIF gene.

10   The inhibitory nucleic acids can also be targeted
against mRNA. In this approach, the inhibitory nucleic acids
are designed to hybridize to a target sequence in MIF mRNA
and, thereby, interfere with its translation. Translation of
mRNA is inhibited if there is a measurable decrease in the

15 amount of MIF protein produced by the cell after treatment.
Inhibiting translation of an mRNA results in suppression of
the cellular function carried out by the encoded protein. For
example, an inhibitory nucleic acid complementary to regions
of c-myc mRNA inhibits c-myc protein expression in a human

20 promyelocytic leukemia cell line, HL60, which over-expresses
the c-myc proto-oncogene. *See* E.L. Wickstrom et al., (1988)
PNAS (USA), 85:1028-1032 and Harel-Bellan et al., (1988) *Exp.
Med.*, 168:2309-2318. Maier et al., (1990) *Science*, 249:1570-
74 showed how to suppress interleukin-1α activity using

25 unmodified antisense oligonucleotides. As described in Helene
and Toulme, inhibitory nucleic acids targeting mRNA have been
shown to work by several different mechanisms in order to
inhibit translation of the encoded protein(s).

          In a preferred embodiment, this invention is
30 directed to methods in which the inhibitory nucleic acid is
directed against a target sequence in MIF mRNA. Inhibitory
nucleic acids for inhibiting the translation of mRNA can have
at least about 15 nucleotides. They also can be as long as
the entire target mRNA. In one embodiment of the invention,

35 the inhibitory nucleic acid is about 30 nucleotides long.

          The activity of an inhibitory nucleic acid depends,
in part, on the location of the target sequence in the mRNA.
Inhibitory nucleic acids that hybridize to MIF mRNA at the

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translation start site, i.e., that are directed against a
sequence that includes the AUG start codon, are particularly
effective in inhibiting translation of the MIF mRNA. In
particular, this invention provides the antisense inhibitory
5   nucleic acid having the sequence GATGAACATA GGCATGGTGG
CGGAGAGACT [SEQ ID NO:3]. The underlined sequence, CAT,
hybridizes to the target ATG start codon and, thereby, the
polynucleotide spans the start codon. Other antisense
inhibitory nucleic acids directed against the start codon
10   include fragments of the aforementioned antisense nucleic acid
of at least about 15 nucleotides, as well as other nucleic
acids having a sequence that hybridizes to a sequence of about
15 nucleotides that span the start codon in the MIF mRNA
sequence. As used herein, the verb "to have," when describing
15   the sequence of a nucleic acid molecule, is used in the narrow
sense to mean that the nucleic acid molecule has the sequence
of nucleotides given without flanking sequences. Inhibitory
nucleic acids directed against other parts of the MIF mRNA,
particularly the 3' untranslated region, are less effective
20   and, therefore, are to be avoided.
                    The inhibitory nucleic acids introduced into the
cell can also encompass fragments of the "sense" strand of the
gene or mRNA to trap or compete for the enzymes or binding
proteins involved in mRNA translation. Of course, these
25   fragments do not encode functional proteins or the target
function would not be inhibited. See Helene and Toulme.
Accordingly, this invention is directed to methods in which
the inhibitory nucleic acid includes sequences from the sense
strand of the MIF gene or MIF mRNA.
30   Also, this invention provides methods in which the
inhibitory nucleic acids induces chemical inactivation or
cleavage of the target genes or mRNA. Chemical inactivation
can occur by the induction of crosslinks between the
inhibitory nucleic acid and the target nucleic acid within the
35   cell. Alternatively, irreversible photochemical reactions can
be induced in the target nucleic acid by means of a
photoactive group attached to the inhibitory nucleic acid.
Other chemical modifications of the target nucleic acids

induced by appropriately derivatized inhibitory nucleic acids
can also be used. For example, the inhibitory nucleic acid
can be attached to manganese or ferric ions. Thus, this
invention is directed to methods wherein the inhibitory
5   nucleic acid is attached to an agent that effects chemical
inactivation of MIF mRNA.

Cleavage, and therefore inactivation, of the target
nucleic acids can be effected by attaching a substituent to
the inhibitory nucleic acid which can be activated to induce
10   cleavage reactions. The substituent can be one that effects
either chemical, photochemical or enzymatic cleavage.

Alternatively cleavage can be induced by the use of
ribozymes or catalytic RNA. In this approach, the inhibitory
nucleic acids would contain either naturally occurring RNA
15   (ribozymes) or synthetic nucleic acids with catalytic
activity. This invention also provides methods in which the
inhibitory nucleic acid cleaves MIF mRNA. Bratty et al.,
(1992) *Biochim. Biophys. Acta.*, 1216:345-59 (1993) and
Denhardt, (1992) *Ann. N.Y. Acad. Sci.*, 660:70-76 describe
20   methods for making ribozymes.

One can synthesize inhibitory nucleic acids for use
in the methods of this invention by any means known to the art
including, for example, chemical synthesis, PCR and expression
from expression vectors. Nucleic acid synthesis methods are
25   well known to those of skill in the art. Inhibitory nucleic
acids are chemically synthesized according to the solid phase
phosphoramidite triester method first described by S.L.
Beaucage and M.H. Caruthers, (1981) *Tetrahedron Letts.*,
22(20):1859-1862 using an automated synthesizer, as described
30   in D.R. Needham-VanDevanter et al., (1984) *Nucleic Acids Res.*,
12:6159-6168. Purification of nucleic acids is by either
native acrylamide gel electrophoresis or by anion-exchange
HPLC as described in J.D. Pearson and F.E. Regnier, (1983) *J.
Chrom.*, 255:137-149.

35   Inhibitory nucleic acids can also be produced by
using polymerase chain reaction (PCR) technology. Appropriate
primers and probes for amplifying the nucleic acid region of
choice are generated from analysis of the DNA sequences.

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Nucleic acid primers complementary to the two 3' borders of
the DNA region to be amplified are synthesized. The
polymerase chain reaction is then carried out using the two
primers. See *PCR Protocols: A Guide to Methods and*

5   *Applications* (Innis, M, Gelfand, D., Sninsky, J. and White,
T., eds.), Academic Press, San Diego (1990). The PCR
amplified DNA can then be denatured and the antisense strand
isolated by standard techniques known to those of skill in the
art. See Sambrook, et *al*., *supra*.

10   The sequence of the inhibitory nucleic acids can be
verified using the chemical degradation method of Maxam and
Gilbert, 1980, in Grossman, L. and Moldave, D., eds. Academic
Press, New York, *Methods in Enzymology*, 65:499-560.

            The inhibitory nucleic acids can be conventional

15 nucleic acids, or are more commonly nucleic acids having
properties which make them more desirable for inhibitory
nucleic acid activity. For example, they can be made
resistant to nucleases or more capable of specific binding to
the desired target sequences. The specific binding can be

20 effected by providing inhibitory nucleic acids having
sequences which result in conventional base-pairing, or which
recognize double-stranded DNA by binding to the major or minor
grooves which are present in the DNA double helix.
Alternatively, the inhibitory nucleic acids, in either single

25 stranded or duplex form, can recognize target protein.

            Whatever the ultimate strategy, it is desirable to
provide oligomers with physiological properties which render
them more effective. The general approach to constructing
various nucleic acids useful in inhibitory nucleic acid

30 therapy has been reviewed by A.R. Vander Krol et *al*. (1988),
Biotechniques 6:958-976, and by C.A. Stein et *al*., (1988)
Cancer Res. (1988) 48:2659-2668. *See* also
*Oligodeoxynucleotides: Antisense Inhibitors of Gene
Expression*, Cohen, J.S., editor, MacMillan Press, London,

35 pages 79-196 (1989), and Antisense RNA and DNA, (1988), D.A.
Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring
Harbor, NY.

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There are a number of approaches described in the art whereby modified nucleic acids are used in these expanded antisense applications. A major impetus for modification of inhibitory nucleic acids is the nuclease susceptibility of unmodified single strand nucleic acids.

The phosphodiester linkages of native DNA and RNA molecules are readily degraded by exonucleases present in cells, tissue culture media, serum, blood and other body fluids. In order to provide enhanced stability *in vivo*, through resistance to endogenous nucleases, nucleic acids are synthesized with alternative linkages other than the conventional phosphodiester linkage. Among these are the methylphosphonates wherein one of the phosphorous-linked oxygens has been replaced by methyl phosphorothioates, wherein sulfur replaces one of the oxygens, and various amidates, wherein NH_2 or organic amino derivatives, such as morpholidates or piperazidates, replace an oxygen. Also carbonate and carbamate linkages can be employed, as well as those involving sulfur rather than oxygen as a linking substituent. Other modified internucleotide linkages can be synthesized that are generally nuclease resistant. *See* Miller *et al.*, (1981) *Biochemistry* 20:1874-1880, Letsinger *et al.*, (1986) *Nucleic Acids Res.* 14:3487-3499, Stein *et al.*, (1988) *Nucleic Acids Res.* 16:3209-3221, and Agrawal *et al.*, (1987) *Tet. Lett.* 28:3539-3542.

Nuclease stability can also be obtained through alternate modifications. For example, Haralambidis *et al.*, (1987) *Tet. Lett.* 28:5199-5202, describe the attachment of a peptide to the 3' hydroxyl terminus of a nucleic acid which was resistant to a 3' exonuclease (snake venom phosphodiesterase) under *in vitro* conditions.

Several additional modifications have been developed to render inhibitory nucleic acids resistant to exo- and endo-nucleases, including sulfur substitution of a deoxyribose phosphodiester oxygen (phosphorothioate nucleotides), and synthesis of the nucleosides in the (α)-anomeric configuration rather than the natural (β)-anomers. For example, the phosphorothioate modification of inhibitory nucleic acids is

used. This form, although resistant to most nucleases, does
not interfere with RNase H cleavage of endogenous mRNA. RNase
H degradation of the endogenous mRNA component of an
(antisense) DNA:RNA hybrid can significantly amplify the

5   inhibition of mRNA translation by the inhibitory nucleic acid
caused by stearic hinderance.

The stability, nuclease resistance, and efficiency
of the inhibitory nucleic acids can be improved by coupling an
intercalating agent such as 2-methoxy, 6-chloro,

10   9-aminoacridine to the 3' or 5' end. The acridine conjugate
can also promote the passage of the inhibitory nucleic acid
out of the endocytic compartment and into the cytoplasm.
Therefore, preferably, acridine-conjugated phosphothiorate
antisense oligodeoxyribonucleotides with specificity for key

15   activation and effector molecules in the targeted autoreactive
T cells are constructed.

Intercalators and chelators which enhance the
ability of the nucleic acid to bind the target DNA or RNA can
also be introduced as a modification. These substituents can

20   be attached to the 5' end of preconstructed nucleic acids
using amidite or H-phosphonate chemistry, as described by K.K.
Ogilvie et al. (1987) *Pure and Appl. Chem.*, 59:325, and by
B.C. Froehler (1986) *Nucleic Acids Res.* 14:5399.
Intercalators can also be attached to the 3' end of oligomers,

25   for example as described by U. Asseline et al. (1989), *Tet.
Lett.*, 30:2521.

In addition, other substituents can be bound to the
3' end of oligomers by alternate methods. For example,
disulfides have been used to attach various groups to the 3'

30   terminus, as described by R. Zuckerman et al., (1987) *Nucleic
Acids Res.* 15:5305. It is known that nucleic acids which are
substituted at the 3' end show increased stability and
increased resistance to degradation by exonucleases (G.
Lancelot et al. (1985) *Biochemistry* 24:2521; U. Asseline et

35   al. (1984), *Proc. Natl. Acad. Sci. USA,* 81:3297).

Nucleic acids also can be synthesized containing a
variety of pseudonucleotides or pseudonucleosides which can
confer desirable properties such as nuclease resistance. The

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pseudonucleotides or pseudonucleosides can also be used as a
means to conjugate other molecules to the nucleic acid, such
as interchelators. See International Patent Application, No.
WO 91/13080, Lin, et al. entitled "Pseudonucleosides and
5   Pseudonucleotides and their Polymers".

The methods of this invention contemplate a variety
of means for delivering the inhibitory nucleic acid to the
cell including, for example, direct uptake of the molecule by
the cell from solution, facilitated uptake through liposome
10   vectors and intracellular expression from an expression
cassette.

One can provide a cell with an inhibitory nucleic
acid by contacting the cell with a soluble inhibitory nucleic
acid, for example, in the culture medium in vitro or in the
15   circulatory system, interstitial fluid or tissue mass in vivo.
Soluble inhibitory nucleic acids present in the external
milieu have been shown to gain access to the cytoplasm and
inhibit translation of specific mRNA species.

In another embodiment, this invention provides
20   expression vectors having an expression control sequence
operatively linked to a nucleic acid sequence that encodes an
inhibitory nucleic acid that inhibits expression of MIF. An
expression control sequence is operatively linked to a nucleic
acid sequence when it directs the transcription and
25   translation of that sequence in an appropriate host cell.
This includes provision of appropriate start and stop codons.

The choice of expression vectors useful in this
invention depends on their intended use. Expression vectors
must, of course, contain expression and replication signals
30   compatible with the host cell. Expression vectors useful in
this invention include viral vectors, such as retroviruses,
adenoviruses and adeno-associated viruses; plasmid vectors;
cosmids; liposomes and the like. Viral and plasmid vectors
are preferred for transfecting mammalian cells.

35   Appropriate expression control sequences for
mammalian cells include, for example, the SV40 promoter, the
RSV (Rous sarcoma virus) promoter and the CMV
(cytomegalovirus) promoter.

The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art. Sambrook et al., (1989) *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, and *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) provide many useful protocols.

Methods of transfecting genes into mammalian cells and obtaining their expression also are well known to the art. See, e.g., *Methods in Enzymology*, vol. 185, Academic Press, Inc., San Diego, CA (D.V. Goeddel, ed.) (1990) or M. Krieger, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, New York, NY, (1990).

This invention also provides methods for inhibiting the activity of MIF in a cell by inhibiting the binding of MIF to Rb protein. One can inhibit the binding of MIF to Rb protein by using compounds that mimic the binding surface of Rb and thereby bind to MIF. These compounds can be, for example, peptides or other organic molecules. The compounds can also be antibodies or engineered binding fragments of antibodies, such as Fab fragments. Small molecules can spontaneously enter cells, while larger molecules may require vectors, such as liposomes, to gain entry.

One can identify such compounds by testing for the ability of the drug to inhibit binding of MIF to Rb protein *in vitro*. Such a test can involve, for example, incubating a sample mixture of the compound to be tested and either MIF or Rb protein, adding Rb protein or MIF, respectively, to the sample mixture, and determining whether the binding of MIF to Rb protein has been inhibited compared to a control sample. Peptides can be identified by screening a peptide library.

In one aspect, methods of inhibiting the activity of MIF are useful in therapeutic treatments for inhibiting the growth of malignant cells in an individual. These treatments involve administering to the individual an effective amount of

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an agent that inhibits the mitogenic activity of MIF in the
cells.

Agents useful in these methods include, for example,
an inhibitory nucleic acid of this invention or a compound
5   that inhibits binding of MIF to Rb protein. In particular,
this invention contemplates the use of expression vectors,
preferably retroviral vectors, for delivery of the inhibitory
nucleic acid molecules of this invention. Methods for
delivering nucleic acids as part of genetic therapy are known
10   in the art, as described above. In one embodiment of the
invention, one can administer the agent by direct injection
into the tumor. One can administer compounds that inhibit the
mitogenic activity of MIF in the form of pharmaceutical
compositions including the compound and a pharmaceutically
15   acceptable carrier.

For systemic administration, injection is preferred,
including intramuscular, intravenous, intraperitoneal, and
subcutaneous injection. Suitable formulations for injection
are found in *Remington's Pharmaceutical Sciences*, Mack
20   Publishing Company, Philadelphia, PA, 17th ed. (1985). A
variety of pharmaceutical compositions comprising agents of
the present invention and pharmaceutically effective carriers
can be prepared. The pharmaceutical compositions are suitable
in a variety of drug delivery systems. For a brief review of
25   present methods of drug delivery, *See*, Langer, (1990) *Science*
249:1527-1533.

The agents of the present invention can be prepared
as formulations in pharmaceutically acceptable media, for
example, saline, phosphate buffered saline, Hank's solution,
30   Ringer's solution, dextrose/saline, glucose solutions and the
like. The compositions can contain pharmaceutically
acceptable auxiliary substances as required to approximate
physiological conditions, such as buffering agents, tonicity
adjusting agents, wetting agents, detergents and the like.
35   Additives can also include additional active ingredients such
as bactericidal agents, or stabilizers.

Systemic administration can also be by transmucosal
or transdermal means, or the agents can be administered

orally. For transmucosal or transdermal administration,
penetrants appropriate to the barrier to be permeated are used
in the formulation. Such penetrations are generally known in
the art, and include, for example, for transmucosal

5   administration, bile salts and fusidic acid derivatives. In
addition, detergents can be used to facilitate permeation.
Transmucosal administration can be through nasal sprays, for
example, or using suppositories. For oral administration, the
agents are formulated into conventional oral administration

10   such as tablets, capsules and tonics.
            In preparing pharmaceutical compositions of the
present invention, it can be desirable to modify the complexes
of the present invention to alter their pharmacokinetics and
biodistribution. For a general discussion of

15   pharmacokinetics, See, Remington's Pharmaceutical Sciences,
supra, Chapters 37-39. A number of methods for altering
pharmacokinetics and biodistribution are known to one of
ordinary skill in the art (See, e.g., Langer, supra).
Examples of such methods include protection of the complexes

20   in vesicles composed of substances such as proteins, lipids
(for example, liposomes), carbohydrates, or synthetic
polymers.
            For example, inhibitory nucleic acids can be
incorporated into liposomes in order to enhance their

25   pharmacokinetics and biodistribution characteristics.
Liposome charge is an important determinant in liposome
clearance from the blood, with negatively charged liposomes
being taken up more rapidly by the reticuloendothelial system
(Juliano, (1975) Biochem. Biophys. Res. Commun. 63:651) and

30   thus having shorter half-lives in the bloodstream. Liposomes
with prolonged circulation half-lives are typically desirable
for therapeutic and diagnostic uses. For instance, liposomes
which can be maintained from 8, 12, or up to 24 hours in the
bloodstream are particularly preferred.

35   A variety of methods are available for preparing
liposomes, as described in, e.g., Szoka et al., (1980) Ann.
Rev. Biophys. Bioeng. 9:467, U.S. Pat. Nos. 4, 235,871,
4,501,728 and 4,837,028, all of which are incorporated herein

by reference. Typically, the liposomes are prepared with about 5-15 mole percent negatively charged phospholipids, such as phosphatidylglycerol, phosphatidylserine or phosphatidylinositol. Added negatively charged phospholipids,

5 such as phosphatidylglycerol, also serve to prevent spontaneous liposome aggregating, and thus minimize the risk of undersized liposomal aggregate formation. Membrane-rigidifying agents, such as sphingomyelin or a saturated neutral phospholipid, at a concentration of at least about 50

10 mole percent, and 5-15 mole percent of monosialylganglioside, can provide increased circulation of the liposome preparation in the bloodstream, as generally described in U.S. Pat. No. 4,837,028, incorporated herein by reference. Additionally, the liposome suspension can include lipid-protective agents

15 which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as αtocopherol and water-soluble iron-specific chelators, such as ferrioxianine, are preferred.

Following liposome preparation, the liposomes can be
20 sized to achieve a desired size range and relatively narrow distribution of liposome sizes. Several techniques are available for sizing liposome to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323.

Following the above treatment, the liposome
25 suspension is brought to a desired concentration for use in intravenous administration. This can involve re-suspending the liposomes in a suitable volume of injection medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the

30 suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration and the liposomes can be administered parenterally or locally in a dose which varies according to, e.g., the manner of administration, the drug being delivered, the

35 particular disease being treated, etc.

For pharmaceutical compositions which comprise the agents of the present invention, the dose will vary according to, e.g., the particular agent, the manner of administration,

the particular disease being treated and its severity, the
overall health and condition of the patient, and the judgment
of the prescribing physician.

The pharmaceutical compositions are intended for
5   parenteral, topical, oral or local administration, such as by
aerosol or transdermally, for prophylactic and/or therapeutic
treatment. The pharmaceutical compositions can be
administered in a variety of unit dosage forms depending upon
the method of administration. For example, unit dosage forms
10   suitable for oral administration include powder, tablets,
pills, and capsules.

The pharmaceutical compositions can be administered
intravenously. Thus, this invention provides compositions for
intravenous administration which comprise a solution of the
15   agent dissolved or suspended in an acceptable carrier,
preferably an aqueous carrier. A variety of aqueous carriers
can be used, e.g., water, buffered water, 0.4% saline, and the
like. For instance, phosphate buffered saline (PBS) is
particularly suitable for administration of soluble complexes
20   of the present invention. These compositions can be
sterilized by conventional, well-known sterilization
techniques, or can be sterile filtered. The resulting aqueous
solutions can be packaged for use as is, or lyophilized, the
lyophilized preparation being combined with a sterile aqueous
25   solution prior to administration. The compositions can
contain pharmaceutically acceptable auxiliary substances as
required to approximate physiological conditions, such as pH
adjusting and buffering agents, tonicity adjusting agents,
wetting agents and the like, for example, sodium acetate,
30   sodium lactate, sodium chloride, potassium chloride, calcium
chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid
carriers can be used which include, for example,
pharmaceutical grades of mannitol, lactose, starch, magnesium
35   stearate, sodium saccharin, talcum, cellulose, glucose,
sucrose, magnesium carbonate, and the like. For oral
administration, a pharmaceutically acceptable nontoxic
composition is formed by incorporating any of the normally

employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient.

     For aerosol administration, the complexes are preferably supplied in finely divided form along with a

5   surfactant and propellant. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic,

10   linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene

15   derivatives of these esters. Mixed esters, such as mixed or natural glycerides can be employed. The surfactant can constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquefied propellants are typically gases at

20   ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above can also be employed. In producing the aerosol,

25   a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

     For topical administration, the agents are

30   formulated into ointments, creams, salves, gels as is generally known in the art. See, Remington's Pharmaceutical Sciences, supra.

     In therapeutic applications, compositions are administered to an individual in an amount sufficient inhibit

35   growth of a malignant cell. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient.

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The following Example is intended to illustrate but not limit the invention.

EXAMPLE

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MIF is induced by two growth factors whose effects are signalled through tyrosine kinase receptor pathways (A. Lanahan et al. (1992) *Mol. Cell. Biol.* 12:3919; A. Ullrich and J. Schlessinger (1990) *Cell* 61:203). To determine whether

10   this effect is specific to this class of receptor signaling pathway, the effect of PDGF on MIF expression in NIH 3T3 cells was compared to that of TGFβ1, another growth factor which is mitogenic in these cells and whose effect is signalled through a serine/threonine kinase system (J. Massague (1992) *Cell*

15   69:1067). NIH 3T3 cells were grown serum-free (control) or in the presence of either 10 ng/ml TGFβ1 overnight, or 5 ng/ml PDGF for 6 hours.

For northern blot analysis, total RNA was extracted from growth factor treated or control cells. 10 μg of total

20   RNA was used per lane and the blot was probed with ^{32}P-labelled mouse MIF cDNA (G.J. Wistow et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:1272). Blots were normalized by hybridization with GAPDH cDNA (ATCC, Rockville, MD). Densitometry was performed on a Bioscan scanner (USB,

25   Cleveland, OH).

Treatment with either growth factor increased endogenous MIF mRNA 2- to 4.5-fold over starting levels (Fig. 1). This induction of MIF mRNA occurs between 2 and 6 hours after the onset of treatment with PDGF, consistent

30   with a delayed early response as observed previously (A. Lanahan et al. (1992) *Mol. Cell. Biol.* 12:3919).

Next, a recombinant MIF gene promoter construct was used to determine whether the induction of MIF mRNA occurs by increased transcription in growth factor stimulated cells.

35   The human MIF gene promoter (V. Paralkar and G. Wistow (1994) *Genomics* 19:48) was cloned upstream of the CAT (chloramphenicol acetyltransferase) reporter gene and stably integrated into NIH 3T3 cells. The human MIF promoter (-1075

to +40) was ligated into the HindIII site of pSVOATCAT vector
(S. Lok et al. (1989) *Nucleic Acids Res.* 17:3563) and
transfected by standard calcium phosphate precipitation (C.
Gorman et al. (1983) *Science* 221:551) into NIH 3T3 cells,
5   along with the expression plasmid pSVONeo, containing the
neomycin phosphotransferase gene (G418 resistance) as
selectable marker. Cells were then grown in the presence of
G418 (750 μl/ml) and stably transfected cell clones were
selected and subcultured by ring cloning.
10   For CAT assay, cells were kept serum-free for
24 hours followed by addition of PDGF (109 ng/ml) or TGFβ1
(15 ng/ml). Cells were harvested 12 hours later to obtain
extracts for fluor-diffusion CAT assay (J.R. Neumann et al.
(1987) *Biotechniques* 5:444) and for protein determination.
15   CAT activity was measured and expressed as activity/min/μg
protein. Activity in the absence of serum was used to
normalize the activity obtained during various treatments.
             When multiple lines of stably transformed cells were
treated with either PDGF or TGFβ1, reporter CAT activity was
20   induced 3 to 5 fold (Fig. 2). Thus, the MIF gene is directly
induced by two different growth factor signaling pathways
suggesting that MIF may have an important role common to cells
undergoing mitogenesis stimulated by various agents.
             In order to determine the function of MIF protein in
25   proliferating cells, an antisense strategy was used.
Antisense and control oligodeoxynucleotides were directly
administered to the cell culture medium, an approach which has
previously proved successful for suppression of interleukin-1α
(J.A. Maier et al. (1990) *Science* 249:1570). Four oligos were
30   used:
        aMIF1: GATGAACATA GGCATGGTGG CGGAGAGACT (SEQ ID NO:3);
        sMIF1: AGTCTCTCCG CCACCATGCC TATGTTCATC (SEQ ID NO:4);
        lFIMa: TCAGAGAGGC GGTGGTACGG ATACAAGTAG (SEQ ID NO:5);
        aMIF2: CTCTTATAAA CCATTTATTT CTCCCGGCTG (SEQ ID NO:6).
35   Oligonucleotide aMIF1 is an antisense to mouse MIF mRNA (A.
Lanahan et al. (1992) *Mol. Cell. Biol.* 12:3919; G.J. Wistow et
al. (1993) *Proc. Natl. Acad. Sci. USA* 90:1272) and centered on
the translation start site. Oligonucleotide sMIF1 is the

complement of aMIF1 as a sense control. Oligonucleotide 1FIMa
is the reverse of aMIF1 as a control for composition.
Oligonucleotide aMIF2 is a second antisense oligo with a
similar G+C/A+T ratio to aMIF1, targeted to the

5   polyadenylation signal of mouse MIF. NIH 3T3 cells were grown
in the presence of these oligos and treated with growth
factors as before (Fig. 3A).

Cell proliferation was assayed using ^{3}H-thymidine
incorporation. NIH 3T3 cells in 0.2% serum at a density of

10   1×10^5 per ml were seeded in 24 well tissue culture plates at
0.5 ml per well and treated with growth factors and oligos as
indicated. After 22 hours, ^{3}H-thymidine was added to the
cells and incorporation into DNA was measured for two hours
(D. Danielpour et al. (1989) *J. Cell. Physiol.* 138:79). For

15   recovery of cells from aMIF1 treatment, aMIF1-containing
medium was replaced by medium containing 10% serum after
22 hours. After a further 22 hours, cells were labelled with
^{3}H-thymidine as before. In the presence of aMIF1, DNA
synthesis in response to both PDGF and TGFβ1 was almost

20   completely inhibited while cells grown in the presence of
sMIF1, 1FIMa and aMIF2 showed normal levels of incorporation.
Cells treated with aMIF1 were not dead, as judged by
microscopic inspection, and rapidly regained the ability to
respond to mitogenic stimuli when the antisense oligo was

25   removed by washing with fresh serum-containing medium (Fig.
3A).

Western blot analysis of extracts of cells treated
with PDGF and TGFβ1 in the presence of aMIF1 showed that MIF
protein levels were greatly reduced by antisense treatment

30   (Fig. 3B). This is consistent with the idea that the effect
of antisense treatment is exerted through direct suppression
of MIF mRNA translation.

The levels of cyclins in antisense treated cells
were then examined to localize the effect of MIF suppression

35   in the cell cycle. Cyclins are markers for different parts of
the cell cycle. Thus, cyclin-E is a marker for late G1 and is
a rate-limiting factor for the G1/S transition, cyclin-A is a
marker for S phase and G2 and cyclin-B is a marker for cells

in G2 (M. Ohtsubo and J.M. Roberts (1993) *Science* 259:1908;
C.J. Sherr (1993) *Cell* 73:1059). Cells were grown with or
without aMIF1 as in Fig. 3. Cell extracts were examined by
western blotting with rabbit anti-human cyclin A, B and E
5   antibodies (UBI, Lake Placid, NY) as described above. In
PDGF-treated NIH 3T3 cells in the presence of aMIF1 cyclin-B
and cyclin-A were greatly reduced while cyclin-E was still
detectable (Fig. 4A). This is consistent with cell cycle
arrest in G1 and suggests that MIF is required for entry into
10   S phase.

These results were confirmed by flow cytometry of
cells grown in the presence and absence of aMIF1 (Fig. 4B).
Compared to cells grown in the presence of PDGF, aMIF1 treated
cells "piled up" in G1 and there was a marked loss of cells in
15   S phase.

To corroborate the results of antisense suppression
the role of MIF in the cell cycle was further investigated by
constitutive expression of the recombinant protein. The
coding sequence of mouse MIF cDNA was cloned into pMAMNeo
20   (Clontech, Palo Alto, CA) and transfected into NIH 3T3 cells.
Stable integrants were selected by G418-resistance. Cells
were analyzed by flow cytometry as above. MIF-constitutive
NIH 3T3 cells had significantly higher rates of proliferation
(Fig. 5) than controls and contained diminished populations in
25   G1 relative to S and G2/M (Fig. 5). This shows that G1 is
shortened and that MIF-constitutive cells progress rapidly
past cell cycle checkpoints into S phase and begin to
accumulate in G2/M. Cells were then deprived of serum.
Although control cells ceased proliferation and accumulated in
30   G1, cells constitutively expressing MIF continued to traverse
the cell cycle (Fig. 5). This is very similar to the results
obtained by overexpressing cyclin-E in mammalian fibroblasts
(M. Ohtsubo and J.M. Roberts (1993) *Science* 259:1908). Thus,
by itself, MIF mimics the mitogenic effects of serum,
35   consistent with it being a common target for induction by a
wide variety of mitogenic agents.

Recently, several small proteins have been
identified which inhibit cell cycle progression through

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interaction with cyclins (J. Pines (1994) *Trends Biochem. Sci.* 19:143). The activity of MIF is consistent with it being an example of a new class of small proteins which act as positive regulatory subunits of important cell cycle control systems.

5   Since MIF acts late in G1, candidate targets for such regulatory activity are cyclin-E and its partner CDK2 which are essential for G1/S transition and Rb, the product of the retinoblastoma gene, which is an important inhibitor of G1/S transition (R.E. Hollingsworth, Jr., et al (1993) *Curr. Opin.*

10   *Genet. Dev.* 3:55). The ability of MIF to interact with these potential targets was examined by Western blot analysis of proteins co-precipitated from extracts of serum-treated NIH 3T3 cells by antisera to cyclin-E, CDK2 and Rb. For immunoprecipitation, NIH 3T3 cells grown in the presence of

15   10% serum were lysed as an incubated in binding buffer (25 mM Tris pH 7.4, 50 mM NaCl, 0.5% Sodium deoxycholate, 0.2% NP-40, 0.2% SDS, 1 mM PMSF, 50 μg/ml aproteinin, 50 μM leupeptin) with either agarose conjugated anti-Rb (Santa Cruz Biotech), or CDK2 (UBI) overnight, or with anti-cyclin E (UBI) for

20   1 hour followed by addition of protein A agarose and overnight incubation. Agarose beads were then washed three times in binding buffer and boiled in the presence of SDS PAGE sample buffer to release bound proteins, followed by SDS PAGE and Western blot analysis using ECL detection as above. Some

25   rabbit immunoglobulin-derived material was released from the beads and subsequently detected in all lanes by horse-radish peroxidase linked to goat anti-rabbit immunoglobulin (Vector Labs).

No MIF was detected in immunoprecipitates of

30   cyclin-E or CDK2; however, a band of the size expected for MIF was detected by the MIF antiserum in immunoprecipitates of Rb (Fig. 6A). The same result was obtained with two different antisera to Rb. Under non-reducing conditions, the MIF-reactive co-precipitated band increased in size to about 30kDa

35   (Fig. 6B), in agreement with studies suggesting that MIF is a homodimer in solution (J. Nishihira et al. (1993) *Biochem. Mol. Biol. Int.* 31:841). These results are consistent with

MIF exerting its effect at G1/S through an interaction with an Rb-containing complex.

The effect of antisense oligonucleotides on the growth of cancer cells also was tested. The proliferation of

5   cultured cells transformed with *ras*, *raf*, or *sis* oncogenes was essentially halted when the cells were exposed to aMIF1.

All references referred to herein are incorporated by reference in their entirety.

Although the foregoing invention has been described

10   in detail for purposes of clarity of understanding, it will be obvious that certain modifications can be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1   1.   A method useful for inhibiting the growth of a
2   cell comprising the step of inhibiting the mitogenic activity
3   of macrophage migration inhibitory factor ("MIF") in the cell.

1   2.   The method of claim 1 wherein the step of
2   inhibiting comprises providing the cell with an inhibitory
3   nucleic acid that inhibits expression of MIF.

1   3.   The method of claim 2 wherein the inhibitory
2   nucleic acid is directed against a target DNA sequence in the
3   sense strand of the MIF gene.

1   4.   The method of claim 3 wherein the target DNA
2   sequence spans the site of initiation of transcription.

1   5.   The method of claim 2 wherein the inhibitory
2   nucleic acid is directed against a sequence comprising the
3   boundary between an intron and an exon of MIF pre-mRNA.

1   6.   The method of claim 2 wherein the inhibitory
2   nucleic acid is directed against a target sequence in MIF
3   mRNA.

1   7.   The method of claim 6 wherein the target
2   sequence comprises the translational start site of MIF mRNA.

1   8.   The method of claim 7 wherein the inhibitory
2   nucleic acid has the sequence GATGAACATA GGCATGGTGG CGGAGAGACT
3   [SEQ ID NO:3].

1   9.   The method of claim 2 wherein the inhibitory
2   nucleic acid comprises a sequence from the sense strand of the
3   MIF gene or from MIF mRNA.

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1   10.   The method of claim 2 wherein the inhibitory
2   nucleic acid induces chemical inactivation of the MIF gene or
3   MIF mRNA.

1   11.   The method of claim 2 wherein the inhibitory
2   nucleic acid cleaves MIF mRNA.

1   12.   The method of claim 2 wherein the step of
2   providing the cell with an inhibitory nucleic acid comprises
3   contacting the cell with the inhibitory nucleic acid.

1   13.   The method of claim 2 wherein the step of
2   providing the cell with an inhibitory nucleic acid comprises
3   transfecting the cell with an expression vector comprising an
4   expression control sequence operatively linked to nucleic acid
5   sequence encoding the inhibitory nucleic acid.

1   14.   The method of claim 1 wherein the step of
2   inhibiting comprises inhibiting the binding of MIF to
3   retinoblastoma ("Rb") protein.

1   15.   The method of claim 1 wherein the cell is a
2   culture cell *in vitro*.

1   16.   The method of claim 15 wherein cell growth is
2   stimulated by the activity of a growth factor.

1   17.   The method of claim 15 wherein cell growth is
2   stimulated by the activity of an oncogene.

1   18.   An isolated inhibitory nucleic acid that
2   inhibits expression of macrophage migration inhibitory factor
3   ("MIF") that is directed against a target sequence selected
4   from the group consisting of a target DNA sequence in the
5   sense strand of the MIF gene, a target sequence comprising the
6   boundary between an intron and an exon of MIF pre-mRNA, and a
7   target sequence in MIF mRNA.

1   19. The inhibitory nucleic acid of claim 18 wherein
2   the target sequence comprises the translational start site of
3   MIF mRNA.

1   20. The inhibitory nucleic acid of claim 19 wherein
2   the inhibitory nucleic acid has the sequence GATGAACATA
3   GGCATGGTGG CGGAGAGACT [SEQ ID NO:3].

1   21. The inhibitory nucleic acid of claim 19 that
2   cleaves MIF mRNA.

1   22. An expression vector comprising an expression
2   control sequence operatively linked to a nucleic acid sequence
3   that encodes an inhibitory nucleic acid that inhibits
4   expression of macrophage migration inhibitory factor ("MIF")
5   and that is directed against a target sequence selected from
6   the group consisting of a target DNA sequence in the sense
7   strand of the MIF gene, a target sequence comprising the
8   boundary between an intron and an exon of MIF pre-mRNA, and a
9   target sequence in MIF mRNA.

1   23. A method useful for inhibiting the growth of a
2   malignant cell in an individual comprising administering to
3   the individual a therapeutically effective amount of an agent
4   that inhibits the mitogenic activity of macrophage migration
5   inhibitory factor ("MIF") in the malignant cell.

1   24. The method of claim 23 wherein the malignant
2   cell expresses the c-ras, c-raf or c-sis oncogene.

1   25. The method of claim 23 wherein the agent is an
2   inhibitory nucleic acid.

1   26. The method of claim 23 wherein the agent
2   inhibits the binding of MIF to retinoblastoma protein.

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1   27.   The method of claim 23 wherein the step of
2   administering the agent comprises injecting the agent into a
3   tumor mass.

1   28.   A pharmaceutical composition comprising an
2   agent that inhibits the mitogenic activity of macrophage
3   migration inhibitory factor ("MIF") in a cell and a
4   pharmaceutically acceptable carrier.

1   29.   The pharmaceutical composition of claim 28
2   wherein the agent is an inhibitory nucleic acid.

1   30.   The pharmaceutical composition of claim 28
2   wherein the agent is a compound that inhibits the binding of
3   MIF to retinoblastoma protein.

1   31.   The pharmaceutical composition of claim 28
2   further comprising a liposome.

FIG. 1.

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FIG. 2.

FIG. 3A.

PDGF   TGF β$_1$

14 kD →

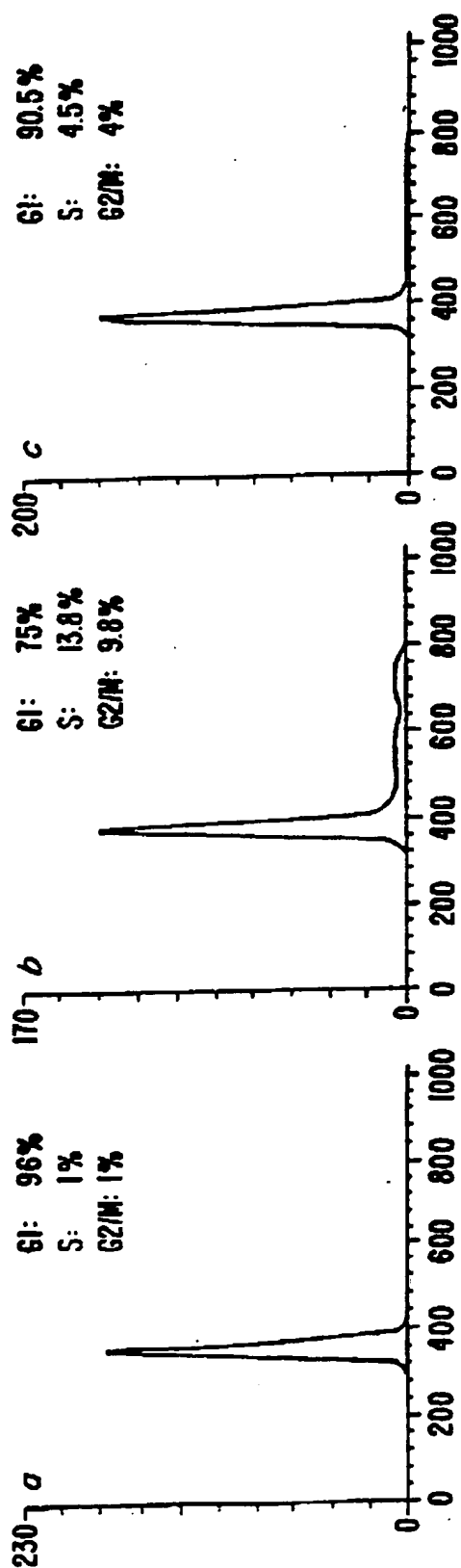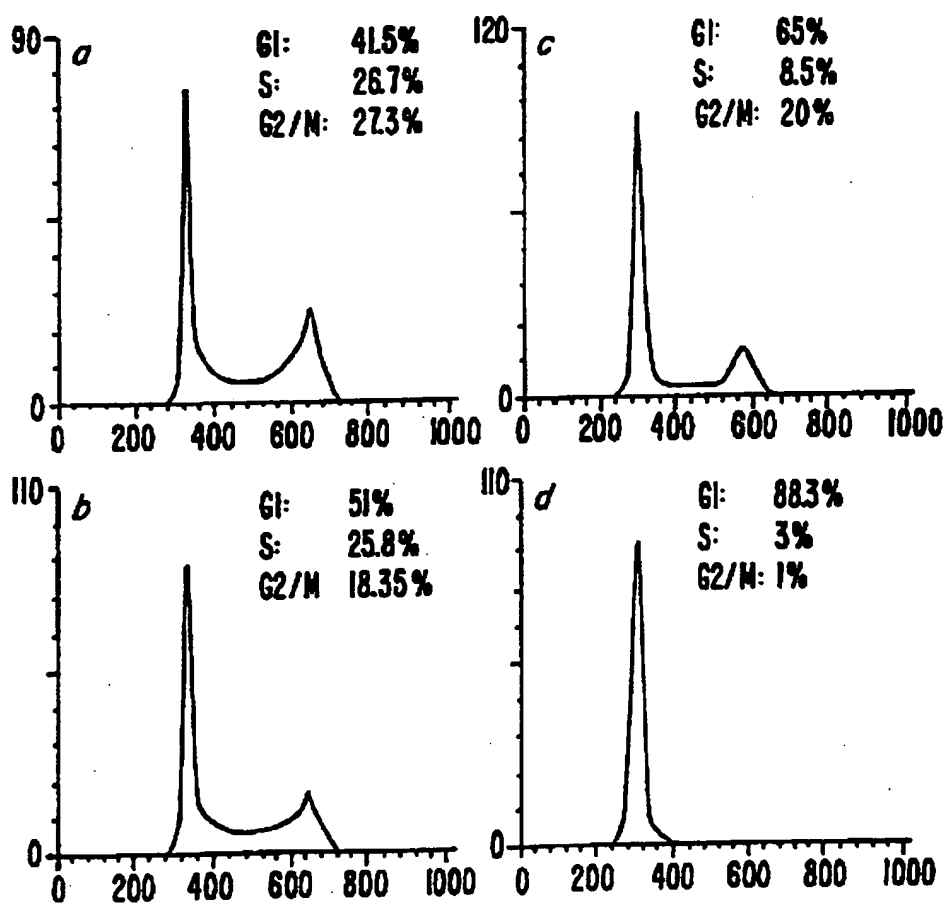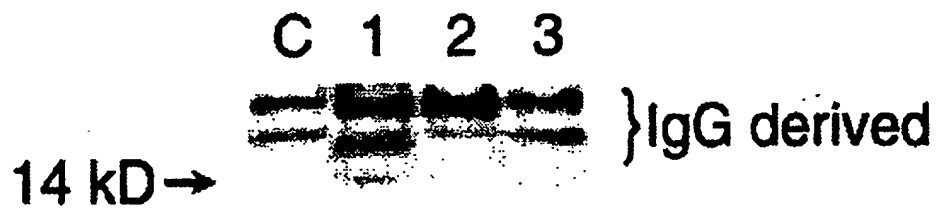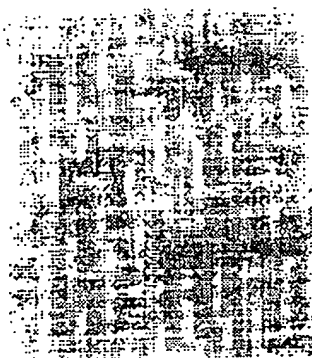aMIF-1   −   +   −   +
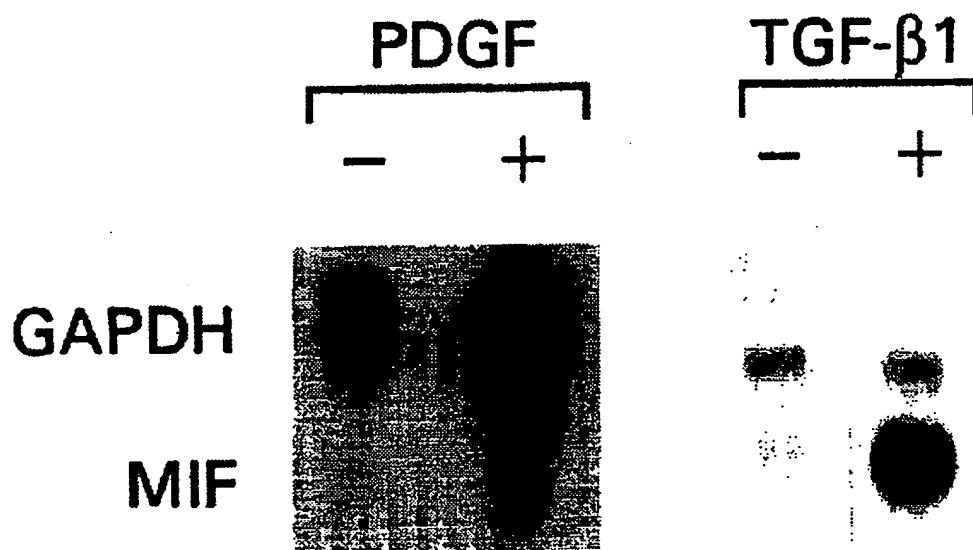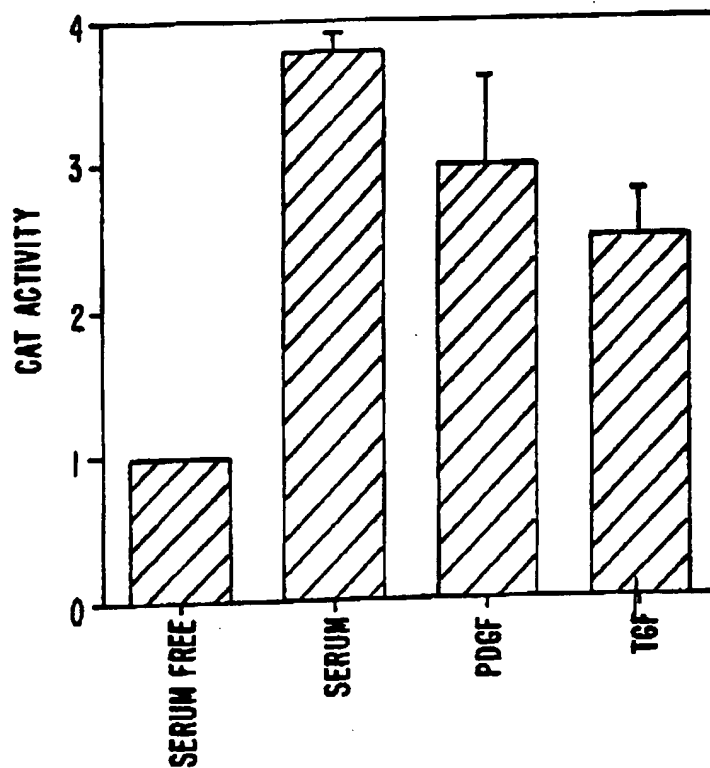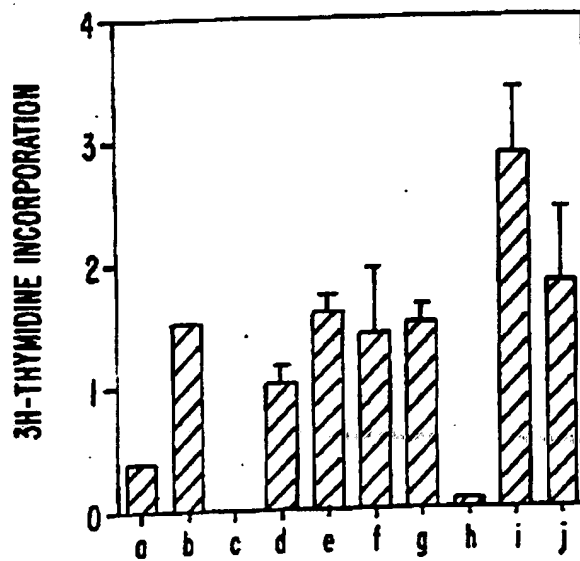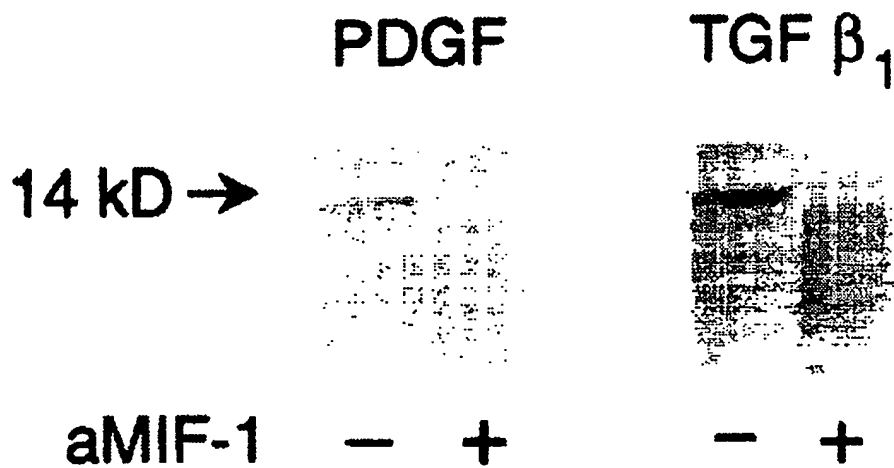
FIG. 3B.
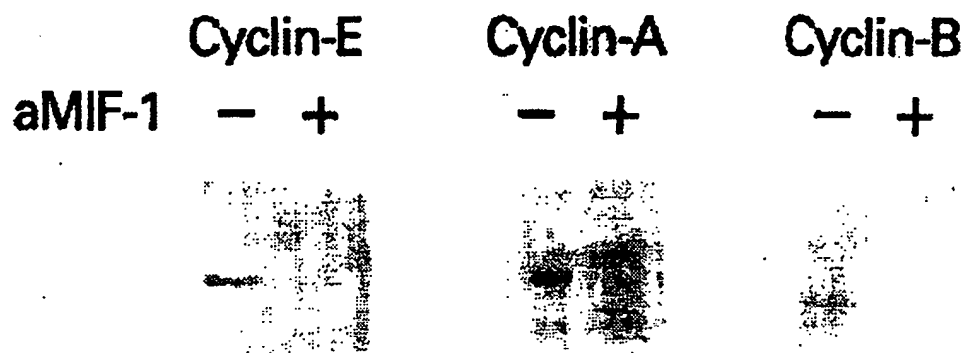
FIG. 4A.

FIG. 4B.

FIG. 5.

FIG. 6A.

C 1

— IgG derived

←30kD

FIG. 6B.